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Research Article

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Protective effect of radiofrequency exposure against menadione-induced oxidative DNA damage in human neuroblastoma cells: The role of exposure duration and investigation on key molecular targets

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Abstract

In our previous studies, we demonstrated that 20 h pre-exposure of SH-SY5Y human neuroblastoma cells to 1950 MHz, UMTS signal, at specific absorption rate of 0.3 and 1.25 W/kg, was able to reduce the oxidative DNA damage induced by a subsequent treatment with menadione in the alkaline comet assay while not inducing genotoxicity per se. In this study, the same cell model was used to test the same experimental conditions by setting different radiofrequency exposure duration and timing along the 72 h culture period. The results obtained in at least three independent experiments indicate that shorter exposure durations than 20 h, that is, 10, 3, and 1 h per day for 3 days, were still capable to exert the protective effect while not inducing DNA damage per se. In addition, to provide some hints into the mechanisms underpinning the observed phenomenon, thioredoxin-1, heat shock transcription factor 1, heat shock protein 70, and poly [ADP-ribose] polymerase 1, as key molecular players involved in the cellular stress response, were tested following 3 h of radiofrequency exposure in western blot and qRT-PCR experiments. No effect resulted from molecular analysis under the experimental conditions adopted.

KEYWORDS

co-exposure, in vitro study, protective effect, radiofrequency

1 | INTRODUCTION

Over several decades, the scientific research into the evaluation of possible adverse effects deriving from radiofrequency (RF) exposure has been carried out with conflicting results in the different lines of evidence of epidemiological, experimental (in vitro, in vivo, human), and mechanistic studies. The general opinion is that the health risk for humans is weak, although the need of good quality additional studies is always highlighted (IEEE, 2019; International Commission on Non-Ionizing Radiation Protection [ICNIRP], 2020; Scientific Committee on Emerging and Newly Identified Health Risks [SCENIHR], 2015).

The need of methodological quality applies also to the in vitro studies which, if properly carried out, should provide support and strength to the results of epidemiological and in vivo studies, and highlight

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interaction mechanisms of these nonionizing radiation with biological material. In vitro experiments are performed mainly on mammalian cell cultures and aim at investigating the effects on key cellular parameters where the effects of RF alone and in combination with other agents (combined exposure) are addressed. From the analysis of these studies, it appears that, at exposure levels below the current limits set by the ICNIRP (ICNIRP, 2020), the results are inconsistent: some studies report effects while other do not. Recently, the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) of the European Commission, by relying on good quality narrative reviews, systematic reviews, and meta-analysis, concluded that there are no consistent effects due to RF exposure on calcium signaling, on apoptosis, on genotoxicity and epigenetics, whereas the cellular oxidative balance may likely be affected, although its correlation with possible adverse effects is not clear (Scientific Committee on Health, Environmental and Emerging Risks [SCHEER], 2023).

Useful indications of potential mechanisms by which RF exposure could affect cellular homeostasis and induce cellular effects can be gained by investigating the effects of combined exposure to RF and other physical or chemical agents. Several protocols for the administration of the agent for co-exposure (before, concurrent, or after RF exposure) have been applied in the published studies, and the results varied greatly based on the cell type, the administration protocol of the agent for co-exposure, and on the RF exposure parameters (Scientific Committee on Emerging and Newly Identified Health Risks [SCENIHR], 2015).

For many years, our research group has been involved in the study of the combined exposure of mammalian cells to RF and other physical or chemical agents. We demonstrated that RF exposure, with several frequencies, signals (2G, 3G, and 4G technologies), and specific absorption rate (SAR) values in almost all cases did not induce biological effects when administered alone. Conversely, RF pre-exposure of several cell models resulted effective in providing protection against the damaging effects of subsequent chemical or physical treatments. We observed that the effect strictly depended on the experimental conditions adopted, both biological and electromagnetic, but the only common characteristic of our studies was 20 h RF exposure (Falone et al., 2018; Romeo et al., 2020; Sannino et al., 2009, 2011, 2014, 2019, 2022, 2024; Zeni et al., 2012, 2021). Considering the variety of experimental conditions resulting effective in eliciting the protective effect, we could argue that RF exposure can act as a mild stressor, not capable of inducing measurable effects per se in the endpoints analyzed, but capable of activating a cellular stress response which makes the cells more prone in coping with the damage induced by a subsequent treatment. In

Highlights

- RF exposure of human neuroblastoma cells at 1950 MHz, UMTS signal, does not induce genotoxicity per se at 0.3 and 1.25 W/kg SAR.
- RF exposure reduces the MD-induced genotoxicity in several time windows along the 72 h cell culture period at both SAR levels.
- RF exposure does not alter protein and gene expression of key molecular players involved in cellular stress response in the assay conditions adopted.

particular, in Falone et al. (2018), we evidenced an enhanced antioxidant scavenging efficiency and restored DNA repair capability in human neuroblastoma (SH-SY5Y) cells pre-exposed for 20 h to 1950 MHz, UMTS signal, at 0.3 and 1.25 W/kg SAR and subsequently treated with menadione (MD), a polycyclic aromatic ketone generating reactive oxygen species (ROS) (Galati et al., 2019).

The experimental conditions adopted by Falone et al. (2018) were applied in a more recent investigation where we demonstrated that in SH-SY5Y cells the inhibition of autophagy negated the protective effect of RF exposure (Sannino et al., 2022).

This protective effect resembles the ionizing radiation-induced adaptive response, investigated since several decades. Several mechanisms have been proposed to explain the phenomenon at the cellular level, but they are still a matter of debate. Among them, DNA damage response, redox to maintain cellular homeostasis, and other effector signaling molecules that mediate either cell survival or eliminate the damaged cell and organelle seem the most plausible (Dimova et al., 2008; Stecca & Gerber, 1998; Thathamangalam Ananthanarayanan et al., 2023).

The current study aims to further characterize the RF-induced protective effect by addressing the role of exposure duration and timing and the possible role of key molecular players involved in the cellular stress response. SH-SY5Y cells were pre-exposed to 1950 MHz UMTS signal for either 3 h, 10 h (given in two different time windows along cell culturing), or 1 h/day for 3 days, subsequently treated for 1 h with 10 μM MD and tested for DNA damage by means of the alkaline comet assay.

Moreover, Thioredoxin-1 (TRX1), Heat shock transcription factor 1 (HSF1), Heat Shock Protein 70 (HSP70), and poly [ADP-ribose] polymerase 1 (PARP1) were tested under a selected experimental condition as possible targets of RF exposure in western blot and quantitative real-time polymerase chain reaction (qRT-PCR) experiments which take into consideration the

proposed mechanisms for ionizing radiation-induced adaptive response.

2 | MATERIALS AND METHODS

2.1 | Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Dominique Deutscher. GlutaMAX was from Gibco™ by Thermo Fisher Scientific Inc. Bovine serum albumin (BSA), menadione, N-lauryl sarcosine, triton X-100, and tween-20 were from SIGMA/Merck. Dimethyl sulfoxide (DMSO), Na_2EDTA, and NaOH were from J. T. Baker. NaCl and Tris-HCl were from Carlo Erba Reagents (Cornaredo Milan). Trypan blue staining solution was from Logos Biosystems (Anyang-si). Ethidium bromide, low-melting point agarose, normal-melting agarose and the chemicals not listed elsewhere for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were purchased from Bio-Rad Laboratories. Protease inhibitor cocktail was from SERVA Electrophoresis GmbH. RIPA buffer, PowerUp SYBR Green Master Mix, RNA purification, and reverse transcription kits were from Thermo Fisher Scientific Inc. Primary antibodies for western blot: TRX1 (sc-271281), HSF1 (sc-17757 S), HSP70 (sc-32239), and PARP1 (sc-74470) were from Santa Cruz; β-Tubulin (9F3) Rabbit mAb (HRP Conjugate) was from Cell Signaling; the secondary antibody mouse IgG A90-116P was from BETHYL.

2.2 | Cell cultures and maintenance

Human SH-SY5Y neuroblastoma cells were purchased by ATCC (Cat. No. CRL2266). They were maintained in 4.5 g/L glucose DMEM, supplemented with 10% heat-inactivated FBS, 1% Glutamax, 100 U/mL penicillin, and 100 mg/mL streptomycin and regularly tested for mycoplasma infection. Cells were kept exponentially growing under standard incubator conditions (37°C, 95% air and 5% CO_2), by replacing with fresh medium every 48 h, and by splitting them once a week by 200 mg/mL trypsin-EDTA treatment.

2.3 | Exposure system set up and dosimetry

The RF exposure set up was designed and realized to gain exposure of cell cultures under strictly controlled environmental and electromagnetic conditions, and has been described and sketched in detail in our previous papers (Falone et al., 2018; Zeni et al., 2012). Briefly, a RF generator (E4432B ESG-D; Agilent) provides the 1950 MHz, UMTS signal to a microwave amplifier (MA-LTD, AM38A-0925-40-43). The signal is then split by means of −6 dB power splitter (HP11667A; Hewlett-Packard), and the output signals are sent to two bidirectional power sensors (NRT-Z43; Rohde & Schwarz) to feed two identical WR430 (350 mm long; SAIREM) short-circuited waveguides. For the connection to the feeding sides, two coaxial-to-waveguide adapters (Maury Microwave R213A2; VSWR: 1.05) are used. A Labview code is used to set up the exposure parameters (frequency, desired SAR value, exposure duration and starting time), to drive the signal generator and to monitor and acquire the levels of the incident and reflected powers at the feeding sides of the waveguides. The power level provided by the RF generator is adjusted, if needed, to keep the required SAR constant throughout the whole exposure time. To fulfill the requirements for cell cultures (37°C, 95% air and 5% CO_2 atmosphere), the waveguides are placed inside a cell culture incubator together with a third one, used for sham-exposures.

The two waveguides were previously optimized and characterized through numerical and experimental dosimetry and temperature measurements. The sample aspect and position inside the waveguide were defined through numerical dosimetry to guarantee high efficiency (>70%) and uniformity of SAR distribution (coefficient of variation <30%) inside each sample. Experimental dosimetry was carried out by calorimetric measurements of SAR and confirmed the performance of the applicators. To rule out any thermal effect, temperature measurements were carried out at regular 5 s intervals for 20 h (worst case scenario for the experiments here presented) in separate experiments, using a fiber-optic thermometer (FisoUMI4; FISO Technologies) with a fiber-optic temperature probe (FOT-M/2 m; FISO Technologies) inserted horizontally into the culture medium. In five independent measurements, the temperature never exceeded the instrument accuracy (±0.3°C) (Falone et al., 2018; Romeo et al., 2013).

2.4 | Experimental procedures

To guarantee consistency and reproducibility of the experiments included in the study, the same batch of reagents were used, and cells were employed from passages 3 to 10. For each experimental run/experimental condition, 1×10^6 cells were seeded in 35 mm Petri dishes (Corning Inc.) and grown for 72 h in 3 mL complete medium. MD (10 μM) was given 1 h before harvesting, where required.

Cell cultures were subjected to different RF exposure durations and timing and analyzed for DNA damage in the alkaline comet assay.

In particular, RF exposure at 1950 MHz, UMTS signal (0.3 or 1.25 W/kg SAR) was provided according to the following duration and timing: (a) 3 h exposure from 48 to 51 h; (b) 10 h exposure from 48 to 58 h; (c) 10 h exposure from 53 to 63 h; and (d) 1 h/day for 3 days. Details are provided in Figure 1.

Each independent experimental run included the following samples in duplicate: untreated control (incubator), sham-exposed (Sh), RF-exposed at 0.3 W/kg (0.3 W/kg), RF-exposed at 1.25 W/kg (1.25 W/kg), MD-treated (MD), sham-exposed and MD treated (Sh +MD), RF-exposed at 0.3 W/kg and MD treated (0.3 W/kg+MD), RF-exposed at 1.25 W/kg and MD treated (1.25 W/kg+MD).

Based on the results from comet assay, 3 h at 1.25 W/kg SAR exposure condition was selected for further experiments with western blot and qRT-PCR analysis.

All the procedures were performed blindly, *i.e.* the researchers involved in sample processing were not aware of the exposure/treatment, and data were decoded after completion of the analyses.

At least three independent experiments were carried out for each condition, and the exact number is provided in the figure captions.

2.5 | Assay procedures

2.5.1 | DNA damage analysis

DNA strand breaks were evaluated by applying the alkaline comet assay with modifications to the protocol of (Singh et al, 1988). After detaching the cells with trypsin and counting with the LUNA-II™ automated cell counter (Logos Biosystems, Inc.), 1×10^5 cells were washed with PBS. Test samples were mixed with low-melting agarose (0.5% w/v) before being placed on a microscope slide between a lower layer of normal-melting agarose (1% w/v) and an upper layer of low-melting agarose (0.5% w/v). Cell lysis was performed in 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 1% N-lauryl sarcosine, with 1% triton X-100 and 10% DMSO for 1 h at 4°C. Equilibration and DNA unwinding stages occurred in 300 mM NaOH, 1 mM Na_2EDTA, pH 13 for 40 min at 4°C. After horizontal electrophoresis (30 V; 340 mA, 40 min at 4°C; Amersham Pharmacia Biotech power supply), the slides were washed three times with 0.4 M Tris-HCl pH 7.5 and once with distilled water.

DNA was stained with 12 μg/mL ethidium bromide and examined by an automated image processing (Delta Sistemi) fitted with a Leica DM BL fluorescence microscope (Leica Microsystems) at ×200 magnification. Two slides were prepared for each experimental condition, and 500 nuclei were randomly examined. The percentage of DNA migrated in the comet tail (% DNA in the tail) was used as a measurement of DNA damage (Zeni & Scarfì, 2010).

2.5.2 | Molecular analysis

TRX-1, HSF1, HSP70, and PARP 1 were assessed for protein and gene expression by western blot and qRT-PCR, respectively.

Three technical replicates from three independent experiments were carried out.

Protein expression

SH-SY5Y cells were lysed in RIPA plus protease inhibitor cocktails and centrifuged at 14,000*g* for 15 min to collect the supernatant. Protein concentration was determined using the Bradford assay and the BSA as standard. Twenty micrograms of proteins were

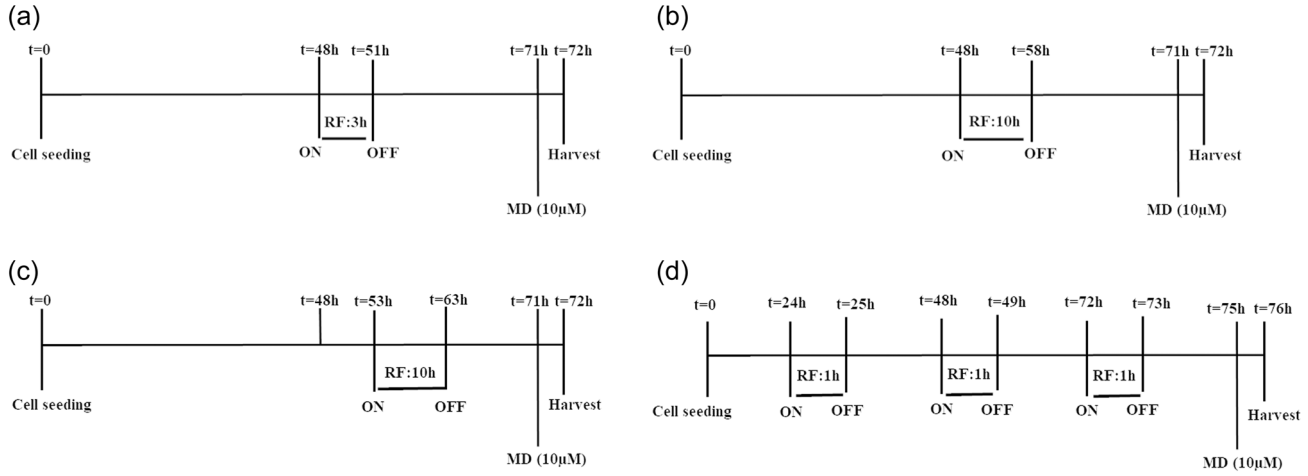

FIGURE 1 Radiofrequency (RF) exposure timing and duration along the 72 h cell culture period. (a) 3 h exposure from 48 to 51 h; (b) 10 h exposure from 48 to 58 h; (c) 10 h exposure from 53 to 63 h, and (d) 1 h/day for 3 days.

TABLE 1 List of primers sequences used for quantitative real-time polymerase chain reaction.

Gene	Forward (5′–3′)	Reverse (5′–3′)
Actin beta	CTGAAGTACCCCATCGAGC	ATAGCACAGCCTGGATAGCAA
HSF1	AGTATAGCCGGCAGTTCTCC	AGATGATGGGTCCAGAGCTG
HSF70	CGACTTTGTCTGTAGGAGCAG	GAATGTGTTAGAGGGAGAAGGTG
PARP1	CGAGTTGTGTCTGAGGACTT	CAGAGTGTTCCAGTCCAGAAT
TRX1	TGGTGAAGCAGATGCAGAGCAAGA	ACCACGTGGCTGAGAAGTCAACTA

TABLE 2 Standard cycling mode for quantitative real-time polymerase chain reaction.

Stage	Temperature (°C)	Duration	Cycles
UDG activation	50	2 min	Hold
Dual-Lock™DNA polymerase	95	2 min	Hold
Denaturation	95	15 s	
Annealing	60	15 s	40
Extension	72	1 min	
Melt curve	Temperature between 60°C and 95°C. Data are collected at each temperature		

separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with EveryBlot blocking buffer for 30 min at RT, the membranes were incubated with primary antibodies: HSP70 (1:800 dilution) for 1 h and TRX-1 (1:1000 dilution), HSF1 (1:300 dilution), and PARP1 (1:300 dilution) overnight at 4°C. The membrane was washed with TBST (Tris Buffered Saline and 0.05% Tween 20) and incubated with the secondary antibody (1:40,000 dilution) for 1 h at RT. Western blot detection was performed by using the Clarity Western ECL Substrate and the ChemiDoc Imaging System (Biorad). The protein bands were quantified by densitometric analysis with Image J software (NIH), and β-tubulin was used as loading control for normalization.

Gene expression

Total RNA was extracted from SH-SY5Y cells by spin column purification with PureLink RNA Mini Kit. RNA samples were quantified by NanoDrop One spectrophotometer (Thermo Fisher Scientific) and quality checked by agarose gel electrophoresis with RunOne System (Embi Tec). Maxima H Minus First Strand cDNA Synthesis Kit was used for genomic DNA elimination and cDNA synthesis. Primer3 program (version 4.1.0; available online at https://primer3.ut.ee/) was employed for designing the PCR primers, listed in Table 1.

The qRT-PCR reaction was set up with 100 ng cDNA, 1× SYBR Green Master Mix, and 300 nM of each forward and reverse primer. The reactions were run by QuantStudio 1 qRT-PCR system (Applied Biosystems by Thermo Fisher Scientific) according to the thermal profile recommended by the supplier and stated in Table 2. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative messenger RNA (mRNA) expression, using sham sample as calibrator and actin beta as reference mRNA (Livak & Schmittgen, 2001). Genes are considered downregulated or upregulated if the fold change is lower than 0.5 or upper than 2, respectively.

2.6 | Statistical analysis

Data for each condition were expressed as mean ± SD. Data were analysed by two tailed unpaired Student's *t* test, and significance was considered at $p < 0.05$ with: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3 | RESULTS

3.1 | RF pre-exposure reduces the menadione-induced DNA damage irrespective of exposure duration and timing

The percentage of DNA in the tail in SH-SY5Y samples exposed/sham exposed at SAR levels of 0.3 and 1.25 W/kg, and co-exposed to MD is presented in Figure 2 for 3 h exposure, 10 h exposures and

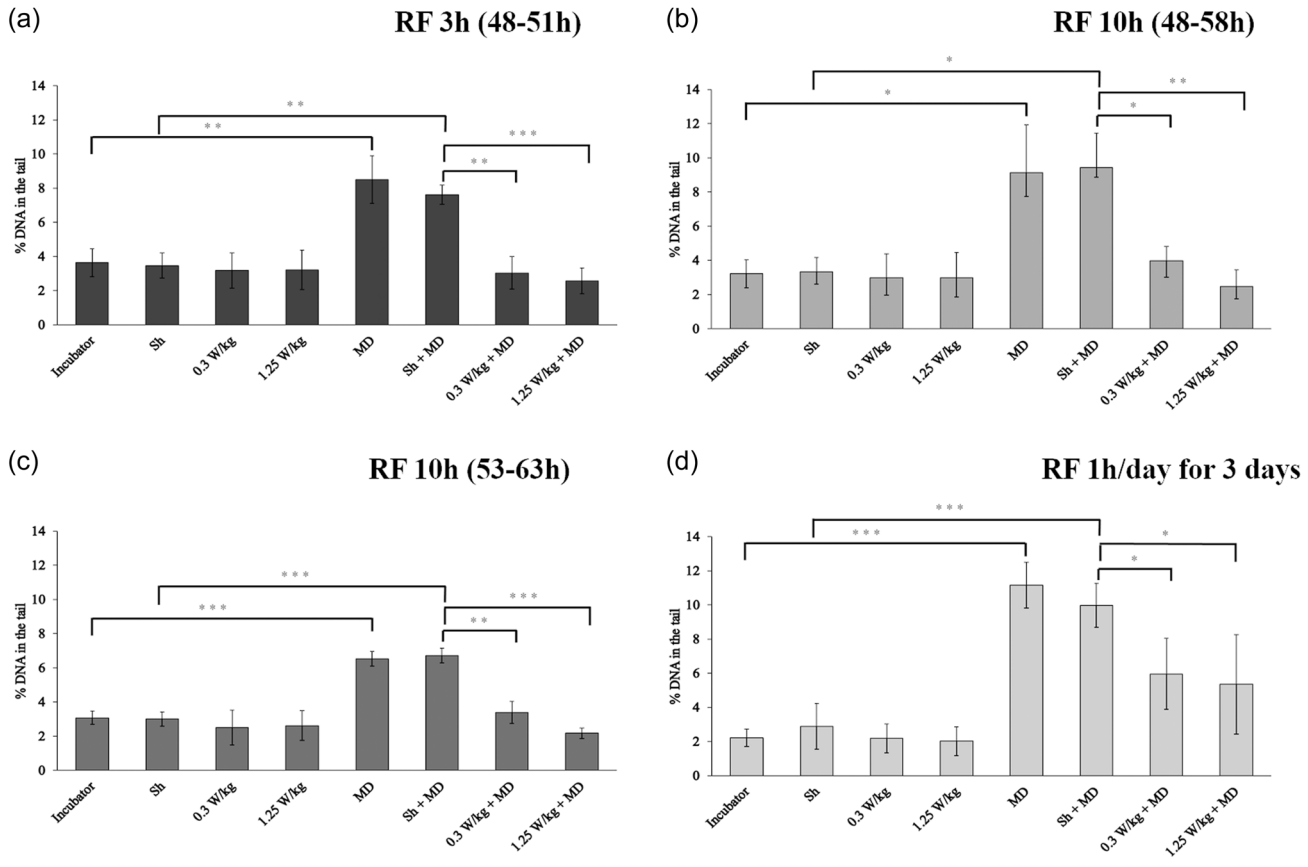

FIGURE 2 DNA damage in SY-SY5Y cells exposed to RF and co-exposed with menadione. % DNA in the tail in SH-SY5Y cells exposed to RF for 3 h, given from 48 to 51 h after seeding (a), 10 h, given from 48 to 58 h (b) or from 53 to 63 h after seeding (c) and for 1 h/day for 3 days, given from 24 to 25 h (Day 1), 48 to 49 h (Day 2) and 72 to 73 h (Day 3) (D). Each data point represents the mean ± SD of three independent experiments, except for (d), where four experiments were carried out. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (two tailed unpaired Student's *t*-test).

intermittent exposure. We found that sham condition altered neither the background level of DNA damage nor the one induced by MD (Sh vs. incubator control; Sh + MD vs. MD), the RF exposure conditions per se did not induce DNA damage (0.3 W/kg vs. Sh; 1.25 W/kg vs. Sh) while MD treatment induced a statistically significant increase in the percentage of DNA in the tail (MD vs. incubator; Sh + MD vs. Sh). These results were gained for all the experimental settings addressing the effects of different RF exposure duration and timing, and for both SAR values investigated.

Interestingly in the case of combined exposures, all the RF exposure conditions here adopted were able to reduce the MD-induced DNA damage as evidenced by comparing RF-exposed cultures and MD-treated (RF + MD) with the sham-exposed and MD-treated ones (Sh + MD). The reduction of DNA % in the comet tail was robust and statistically significant in all the experimental settings, ranging between 40% and 60% and between 46% and 74% for 0.3 and 1.25 W/kg SAR, respectively.

Cell viability was assayed in all the experimental cultures and resulted never below 80%, including the MD-treated ones (trypan blue dye exclusion method; data not shown).

3.2 | The protein and gene expression levels of selected cellular targets resulted unaffected in the examined conditions

The results of WB analysis and qRT-PCR are presented as mean ± SD of three independent experiments in Figures 3 and 4, respectively. Figure 3 includes also a representative western blot image of each target protein. Since sham-exposed cells experienced the same environmental conditions of the exposed ones (except for RF exposure) and the results did not differ from the ones of incubator control, the former were considered as the most appropriate reference control for the analysis of gene expression.

HSF1, HSP70, PARP1, and TRX1 resulted unaffected in both WB analysis and qRT-PCR in all the experimental conditions adopted suggesting that RF exposure, given alone and in combination with MD is not

	Incubator	Sh	1.25 W/kg	MD	Sh + MD	1.25 W/kg + MD
HSF1	0.55±0.4	0.62±0.54	0.55±0.33	0.32±0.11	0.33±0.13	0.23±0.04
HSP70	1.27±0.59	1.15±0.58	1.13±0.85	0.94±0.5	0.85±0.42	0.96±0.59
PARP1	0.89±0.47	0.98±0.6	0.98±0.38	0.97±0.27	1.12±0.19	0.89±0.3
TRX1	0.79±0.19	0.81±0.27	0.56±0.37	0.6±0.32	0.47±0.21	0.5±0.21

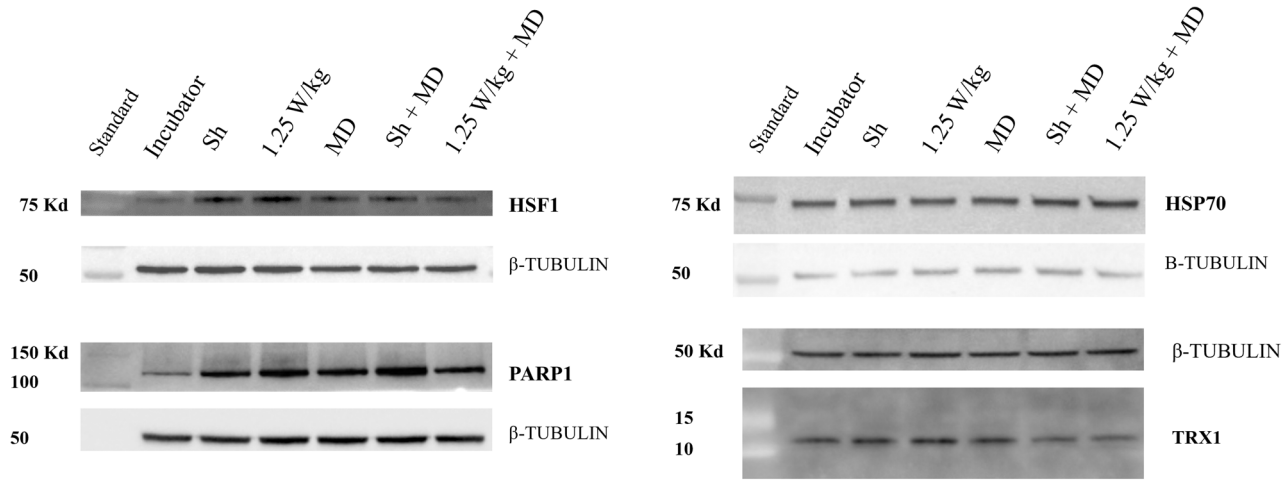

FIGURE 3 Protein expression levels of HSF1, HSP70, PARP1 and TRX in SH-SY5Y cells exposed to RF for 3 h. Each data point represents the mean ± SD from 3 independent experiments. Representative WBs are also presented for each molecular target.

able to modify gene and protein expression of the selected targets ($p > 0.05$ in all cases).

4 | DISCUSSION

A considerable number of in vitro studies has been carried out to look at possible cellular mechanisms by which RF exposure under nonthermal conditions might alter cellular homeostasis and ultimately exert negative effects on human health. To this aim, several cell models have been used, and the effects of both RF exposure alone and in combination with other agents have been investigated on key cellular parameters, mainly related to oxidative stress, cytotoxicity, and genotoxicity. However, given the wide range of RF exposure parameters and experimental protocols for exposure/co-exposure used in the different studies, as well as frequently contradictory results, many of these effects remain controversial. Moreover, studies done to replicate positive results often turned out negative without any methodological explanation for the divergence of results ([SCENIHR], 2015, [SCHEER], 2023). Regarding co-exposure with chemical or physical agents, an increase or a decrease in the cellular damage induced by these agents has been observed in some cases, and the critical role of the sequence of delivery has been highlighted (Kostoff & Lau, 2013). However, further investigations are required to clarify the involvement of RF in the observed effects.

In this study, we applied cell and molecular biology techniques to further elucidate the protective effect previously observed in human neuroblastoma cells pre-exposed for 20 h to 1950 MHz RF and subsequently challenged with MD (Falone et al., 2018; Sannino et al., 2022; Zeni et al., 2021). As a matter of fact, neuronal cells are often used as in vitro model of neuronal function and differentiation in bioelectromagnetics studies also due to the proximity of mobile phones to the head and the electrical activity of the brain.

The experiments have been performed in accordance with the requirements for a good quality in vitro investigations in bioelectromagnetic research to guarantee the control of both electromagnetic and biological parameters in the exposed samples (Paffi et al., 2010; Zeni & Scarfì, 2012).

We evidenced here that 3 h, 10 h, and 1 h/day for 3 days RF exposure resulted in a reduction of the DNA

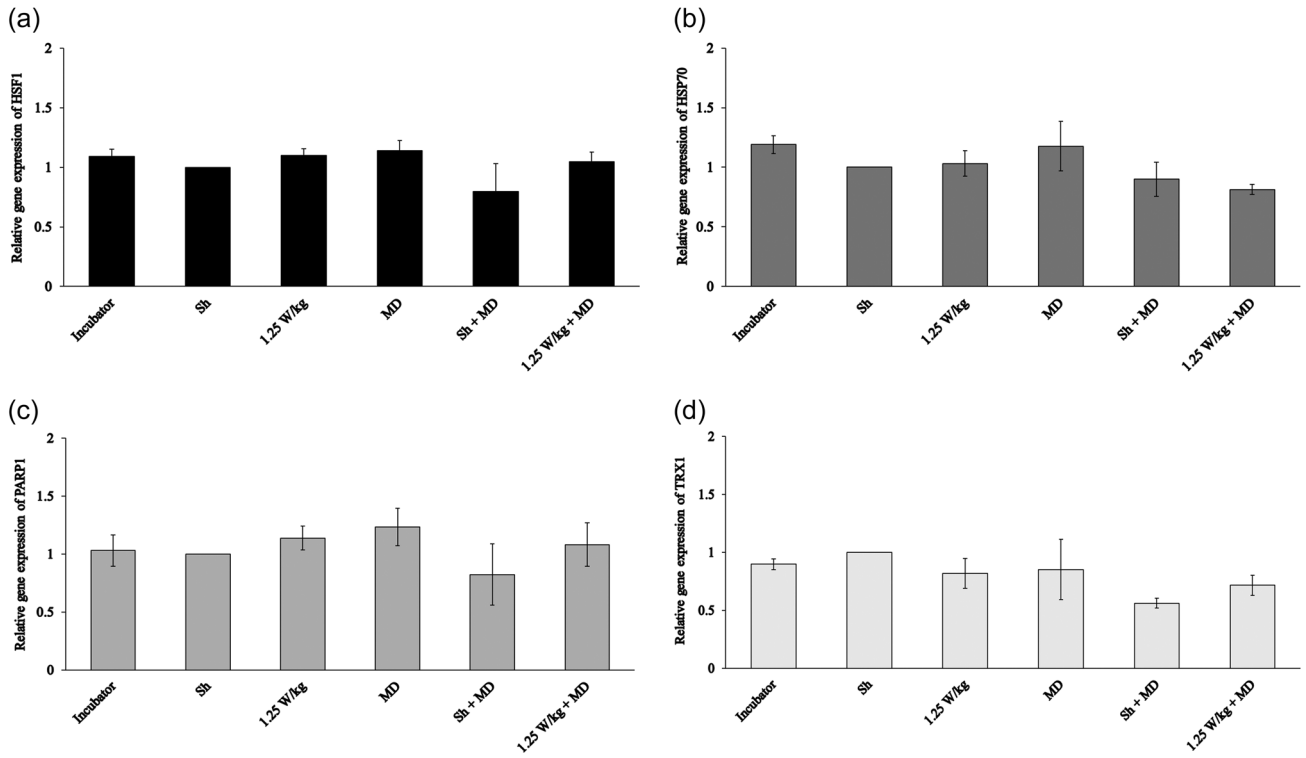

FIGURE 4 Gene expression levels of HSF1 (a), HSP70 (b), PARP1 (c), and TRX (d) in SH-SY5Y cells exposed to RF for 3 h. Each data point represents the mean ± SD from 3 independent experiments.

damage induced by the subsequent treatment with MD, in absence of any alteration of cell viability. These results were comparable to the one induced by 20 h RF exposure and seem profiling an intrinsic capability of RF exposure in activating a cellular stress response mechanism in neuroblastoma cells which, irrespective of the RF exposure duration and timing, is capable of offering protection towards the oxidative DNA damage induced by a subsequent treatment with MD. In order to start exploring a potential mechanism underneath our observations, we focus here on investigating the protein and gene expression levels of key molecular players such as HSF1, HSP70, PARP1, and TRX. HSPs are normally expressed in all cell types in response to environmental stressors and are activated by HSFs. HSF1 is a key player in the cellular stress response which acts as a regulator of stress-protective gene transcription. It is activated in response to stressful conditions through oligomerization and is retained in the nucleus where it binds heat shock elements (HSEs) adjacent to target genes or in distal regulatory elements to promote cell survival through several mechanisms (Åkerfelt et al., 2010; Gomez-Pastor et al., 2018). HSF1 is the main responsible of HSP70 transcription, while PARP1 is among the HSF1 interactive proteins (Fujimoto et al., 2018). PARP is a multifunctional regulator of chromatin structure, transcription, and DNA repair (Gupte et al., 2017). Moreover, it is also reported in the literature that HSP70 is involved in protecting cells against primary DNA damage induced by several genotoxic agents or in protecting DNA from further damage (Abe et al., 1995). The redox protein TRX has a protective role against oxidative stress and is ubiquitarian in cytoplasmic environment. Additionally, TRX has been shown to interact with HSF1 during oxidative stress and increase the DNA binding capability of HSF1 (Jacquier-Sarlin & Polla, 1996).

There are several investigations in the literature which address gene and protein expression after in vitro RF exposures. They mainly evaluate the effects on stress proteins including HSP70 with the majority of studies reporting no effect (McNamee & Chauhan, 2009; Miyakoshi, 2013) although the lack of consistent theoretical, as well as experimental, arguments for specific gene and/or protein response patterns after RF exposure makes these investigations very diversified and inconsistent (Vanderstraeten & Verschaeve, 2008). Our research group assessed intracellular and extracellular HSP70 expression in SH-SY5Y cells exposed to 1950 MHz UMTS-modulated RF EMF for 20 h at a SAR of 0.3 W/kg, and reported no change in intracellular HSP70, but observed an increase in extracellular HSP70 (Zeni et al., 2021). Regarding specifically PARP1 involvement, the current result seems in contrast with previous results available in the literature. As a matter of fact, in two different cell models (human peripheral

blood lymphocytes and Chinese hamster lung fibroblasts) exposed for 20 h to 1950 MHz, UMTS signal, and then treated with Mitomycin-C, we demonstrated the possible involvement of PARP by indirect measurements through 3-aminobenzamide treatment which is a PARP inhibitor (Sannino et al., 2019). Direct evidence for the involvement of PARP1 in the protective effect of RF exposure were demonstrated by other research groups in different cell models and under different RF exposure/co-exposure conditions (He et al., 2016, 2017). HSF1 was tested in a recent study by using a bioluminescence resonance energy transfer technique. No evidence of HSF1 activation was detected in human embryonic kidney (HEK 293 T) cells exposed to 1800 MHz CW, GSM, or WiFi-modulated RF-EMF for 24 h at a SAR of 1.5 or 6.0 W/kg when cell culture temperature was controlled (Poque et al., 2021). To the best of our knowledge, experimental investigations which address gene and protein expression of TRX under RF exposure are not available in the literature although activation of the ROS system, and the consequent oxidative stress is deemed to be the main critical condition that could provide evidence of a mechanism by which RF exposure might affect cellular homeostasis (Schuermann & Mevissen, 2021). The overall results of the molecular analyses here performed do not trace a specific pathway involving the analyzed targets since negative results have been registered under the examined conditions although we are aware of the limitation of our study design. As a matter of fact, we performed the analysis at the end of culture period, that is, at 21 h after the end of RF exposure when the protective effect was found in the comet assay, and we cannot exclude that PARP1 gene can be activated at different times after RF exposure before the end of culture period. Similarly, we cannot exclude that some changes can occur in the other protein and gene targets here considered if assayed in other time windows between the end of exposure and the MD treatment. Thus, our future investigations will aim at addressing these molecular targets more systematically. Moreover, other molecular targets involved in the cellular stress response will be investigated which likely can underneath the recurrent protective effect induced by pre-exposure to RF against several damaging agents.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

Not applicable.

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